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(71) Applicant:
F. HOFFMANN-LA ROCHE AG
4070 Basel (CH)

(72) Inventors:
• **Babyak, Lyubov Ya.**
290056 Lviv (UA)
• **Bacher, Adelbert**
85748 Garching (DE)
• **Boretskyy, Yuriy R.**
290035 Lviv (UA)
• **Demchyshyn, Vasyl V.**
290040 Lviv (UA)

• **Eberhardt, Sabine**
85737 Ismaning (DE)
• **Fedorovych, Dariya**
290000 Lviv (UA)
• **Luetngen, Holger**
85748 Garching (DE)
• **Richter, Gerald**
85080 Gaimersheim (DE)
• **Van Loon, Adolphus**
4310 Rheinfelden (CH)

(74) Representative: **Braun, Axel et al**
F.Hoffmann-La Roche AG
Patent Department (PLP),
124 Grenzacherstrasse
4070 Basel (CH)

Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **Overproduction of riboflavin in yeast**

(57) The present invention is directed to a yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promoter functional in such yeast strain, a process for the production of riboflavin characterized therein that such a yeast strain is cultured under suitable culture conditions and process for the production of a food or feed composition

EP 0 967 287 A2

Description

[0001] Derivatives of riboflavin (the flavocoenzymes FMN and FAD) are universally required for redox reactions in all cellular organisms. Riboflavin (vitamin B₂) is produced by all plants and by many microorganisms [Demain A.L. Riboflavin oversynthesis. Ann. Rev. Microbiol. 1972, 26, 369]. The compound is not produced in vertebrates. Riboflavin is therefore an essential nutrient for man and animals.

[0002] Riboflavin can be produced by chemical synthesis and by various fermentation procedures using strains of *Bacillus* (e.g. *Bacillus subtilis*), the ascomycetes *Ashbya gossypii* and *Eremothecium ashbyi* [Demain A.L. Riboflavin oversynthesis. Ann. Rev. Microbiol. 1972, 26, 369 and Mitsuda H, Nakajima K., Effects of 8-azaguanine on riboflavin production and on the nucleotide pools in non-growing cells of *Eremothecium ashbyi*. J. Nutr Sci Vitaminol (Tokyo) 1973; 19(3):215-227], various yeast strains such as *Candida guilliermondii*, *Candida famata* [F.W. Tanner, Jr.C. Vojnovich, J.M. Van Lanen. Riboflavin production by *Candida* species. Nature, 1945, 101 (2616):180-181] and related strains, as well as other microorganisms.

[0003] The pathway of riboflavin biosynthesis in yeast is shown in Fig. 1. The precursors for the biosynthesis of the vitamin are guanosine triphosphate (GTP) and ribulose 5-phosphate. One mol of GTP and two mol of ribulose 5-phosphate are required to biosynthetically generate one mol of riboflavin.

[0004] In the yeast, *Saccharomyces cerevisiae*, the biosynthesis of the vitamin requires at least six genes, specifically the genes *RIB1*, *RIB2*, *RIB3*, *RIB4*, *RIB5* and *RIB7* [Oltmanns O., Bacher A., Lingens F. and Zimmermann F.K. Biochemical and genetic classification of riboflavin deficient mutants of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 1969, 105, 3061. In *C. guilliermondii*, the biosynthesis of riboflavin has also been shown to require the products of at least six genes, specifically the genes *RIB1*, *RIB2*, *RIB3*, *RIB4*, *RIB5* and *RIB6* (2). The enzymes specified by these *C. guilliermondii* genes and their roles in the biosynthetic pathway are summarized in Fig. 1. In contrast to the situation in *B. subtilis*, the riboflavin biosynthetic genes are not clustered in the eukaryotes *S. cerevisiae* and *C. guilliermondii*.

[0005] The initial step in the biosynthetic pathway is the opening of the imidazole ring of GTP catalyzed by the enzyme, GTP cyclohydrolase II. The product of this enzyme has been reported to be 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate. This intermediate is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of side chain reduction, ring deamination, and dephosphorylation. The hypothetical enzyme involved in the dephosphorylation of 5-amino-6-ribitylamino 5'-phosphate is still unknown. The conversion of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to 6,7-dimethyl-8-ribityllumazine by the enzyme, 6,7-dimethyl-8-ribityllumazine synthase, requires a second substrate, 3,4-dihydroxy-2-butanone 4-phosphate, which is obtained from ribulose 5-phosphate by the catalytic action of 3,4-dihydroxy-2-butanone-4-phosphate synthase. Finally, 6,7-dimethyl-8-ribityllumazine is converted to riboflavin by a dismutation reaction catalyzed by riboflavin synthase. The sequence of the *RIB1* gene directing the synthesis of GTP cyclohydrolase II, the initial enzyme of the riboflavin pathway, has been established in the yeast, *C. guilliermondii* (4).

[0006] Recombinant strains of *Bacillus subtilis* for the production of riboflavin by fermentation have been described, e.g. in EP 405 370. These strains carry the riboflavin operon under the control of a strong promoter directing the production of the cognate enzymes in large amount. The gene constructs of the riboflavin operon under the control of a strong promoter can be present at one or several different locations on the *B. subtilis* chromosome. The incorporation of an additional gene of the riboflavin pathway under the control of a strong promoter at a separate locus on the *B. subtilis* chromosome has also been shown to increase the yield of riboflavin obtained by fermentation, see EP 821 063.

[0007] Whereas the production of riboflavin by strains of yeasts such as *C. guilliermondii* has been reported, recombinant DNA technology has not been applied for the overexpression of riboflavin biosynthetic genes in *C. guilliermondii* or in related flavinogenic yeasts so far.

[0008] It is therefore an object of the present invention to provide recombinant means which should allow the production of yeast strains which overproduce riboflavin. More specifically it is an object of the present invention to provide a yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promoter functional in such yeast strain and even more specifically such a yeast strain which belongs to the groups of flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation-like a yeast strain which is selected from the following group: *Schwanniomyces*, preferably *Schwanniomyces occidentalis*, *Debaryomyces*, preferably *Debaryomyces hansenii*, *Torulopsis*, preferably *Torulopsis candida*, or, especially *Candida*, preferably *Candida guilliermondii* or *Candida famata* [Logvinenko et al., Ukrainskii Biokhimicheski Zhurnal 61(1), 28-32, 1989; Logvinenko et al., Mikrobiologiya 57(2), 181-186, 1988 and Nakase and Suzuki, Journal of General and Appl. Mikrobiology 31(1), 49-70 (1985)]. It is furthermore an object of the present invention to provide such yeast strains wherein the polypeptide encoding DNA sequence is from yeast, preferably flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation, more preferably *Candida*, e.g. *Candida guilliermondii* or *Candida famata*.

[0009] It is also an object of the present invention to provide such yeast strains wherein the polypeptide encoding DNA

bacteria

yeast strain
into yeast

sequence encodes a protein with GTP cyclohydrolase II activity and is selected from the following DNA sequences:

a) the DNA sequence as shown in Fig. 5 or its complementary strand;

b) DNA sequences which hybridize under standard conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and

c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

[0010] It is furthermore an object of the present invention to provide such yeast strains wherein the promotor is the TEF *S. cerevisiae* promotor. (6)

[0011] It is also an object of the present invention to provide a process for the production of riboflavin characterized therein that a yeast strain as described above is cultured under suitable culture conditions and the riboflavin produced is isolated from the medium or the yeast strain by methods known to the man skilled in the art, and a process for the production of a food or feed composition characterized therein that riboflavin as obtained by such process is mixed with one or more suitable food or feed ingredients by a process known to the man skilled in the art. (7)

[0012] All *C. guilliermondii* strains used in the practice of the present invention are derivatives of the *C. guilliermondii* strain obtained from the American Type Culture Collection (ATCC) under accession No. ATCC 9058 (1): *Candida guilliermondii* (ATCC 9058) has been redeposited as a Budapest Treaty deposit on April 1, 1998 and has been assigned accession No. ATCC 74437. *Candida guilliermondii* is a representative of yeast species which overproduce riboflavin (vitamin B2) under conditions of iron starvation. The group includes also *Schwanniomyces occidentalis*, (or called *Debaryomyces occidentalis*) *Debaryomyces cloeckeri*, *Torulopsis candida* and *Candida famata*. The latter species is used for industrial production of riboflavin. Regarding the taxonomic assignments of yeast species a man skilled in the art knows that these assignments are handled variably by different authors, for example: *Candida famata* - *Debaryomyces hansenii* - *Torulaspora hansenii*, *Candida guilliermondii* - *Pichia guilliermondii* - *Yamadazyma guilliermondii* are used as synonyms. A man skilled in the art knows that microorganisms which can be used for the practice of the present invention, either as host cells or source for the isolation of DNA sequences, are available from depository authorities, e.g. the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) or the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) or any other depository authority as listed in the Journal "Industrial Property" [(1991) 1, pages 29-40]. Pg 9058 C.f.

[0013] DNA sequences useful for the practice of the present invention and encoding a polypeptide with riboflavin biosynthetic activity can be obtained from any microorganism known to produce riboflavin (see above) in form of e.g., genomic or c-DNA sequences by methods known to the man skilled in the art or by using the wellknown PCR-Technology. The principles of the polymerase chain reaction (PCR) method are outlined e.g. by White et al., Trends in Genetics, 5, 185-189 (1989), whereas improved methods are described, e.g. in Innis et al. [PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990)].

[0014] The sequence information needed for the design of the PCR-primers can be obtained from any sequence data base, for example like Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinxton Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin, USA).

[0015] Once such DNA sequences have been obtained they can be expressed in any desirable host and the riboflavin biosynthetic activity of the encoded polypeptide can be determined by any assay known to the man skilled in the art and described, e.g. in Bacher A., G. Richter, H. Ritz, S. Eberhardt, M. Fisher and C. Krieger, Biosynthesis of riboflavin: GTP cyclohydrolase II, deaminase, and reductase. Methods in enzymology 1997; 280: 382-389; K. Kis, R. Volk and A. Bacher, Biosynthesis of riboflavin. Studies on the reaction mechanism of 6,7-dimethyl-8-ribityllumazine synthase. Biochemistry 1995, 34, 2883-2892; Logvinenko EM, Shavlovskii GM, Zakal'skii AE, Kontorovskaia Niu. Properties of 2,5-diamino-4-oxy-6-ribosylaminopyrimidine-5'-phosphate reductase, an enzyme of the second stage of flavinogenesis in *Pichia guilliermondii* yeast Ukr Biokhim Zh 1989 Jul; 61(4): 47-54; G. Richter, M. Fischer, C. Krieger, S. Eberhardt, H. Lüttgen, I. Gerstenschläger and A. Bacher. Biosynthesis of riboflavin. Characterization of the bifunctional deaminase/reductase of *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 1997, 179, 2022-2028; K. Ritsert, D. Turk, R. Huber, R. Ladenstein, K. Schmidt-Bäse and A. Bacher. Studies on the lumazine synthase/riboflavin synthase complex of *Bacillus subtilis*. Crystal structure analysis of reconstituted icosahedral β subunit capsid at 2.4 Å resolution. J. Mol. Biol. 1995, 253, 151-167.

[0016] The DNA sequences used for the practice of the present invention comprise at least one DNA sequence which encodes a polypeptide with riboflavin biosynthetic activity. It is however, understood by the man skilled in the art that also more than one, e.g. all enzymes of the riboflavin biosynthetic pathway can be encoded by such DNA sequences and one or more of these enzymes can be encoded by DNA sequences of different species origin or can be of partial or total

synthetic origin as long as they show at least one desired riboflavin biosynthetic activity. One example of such a DNA sequence is given in Fig. 5 coding for a GTP cyclohydrolase II. However, DNA sequences which hybridize under standard conditions to this DNA sequence and encode such a GTP cyclohydrolase are also useful for the practice of the present invention.

- 5 [0017] "Standard conditions" for hybridization in this context are conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, e.g. by Sambrook et al., "Molecular Cloning" second edition, Cold Spring Harbor Laboratory Press 1989, New York, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, e.g. in Sambrook et al. (s.a.). "Fragment of the
- 10 DNA sequences" means in this context a fragment which codes for a polypeptide still having the enzymatic activity as specified above.

- [0018] For the overexpression of the proteins encoded by the DNA sequences of the present invention these sequences can be linked to promoters which are functional in the desired yeast and are, e.g. the *S.cerevisiae* TEF-promotor (see Example 2) or the *pho5*-promotor [Vogel et al., Mol. Cell. Biol., 2050-2057 (1989); Rudolf and Hinnen, Proc. Natl. Acad. Sci. 84, 1340-1344 (1987)] or the *gap*-promotor or the *aox1*-promotor [Koutz et al., Yeast 5, 167-177 (1989); Sreekrishna et al., J. Basic Microbiol. 28, 265-278 (1988)] or the FMD promotor [Hollenberg et al., EP 299108] or MOS-promotor [Ledeboer et al., Nucleic Acids Res. 13, 3063-3082 (1985)].

- 15 [0019] The DNA sequences useful for the practice of the present invention can also comprise so called "ARS" elements (autonomously replicating sequence) as described, e.g. in Example 1.

TEF promoter
pho5 promoter
gap promoter
aox1 promoter
fmd promoter
ARS
elements

20

Examples

- [0020] If not specifically indicated or referred to by references standard procedures have been used as described, e.g. in Sambrook et al. "Molecular Cloning", (s.a.) and Cregg, J.M., K.J. Barriner, A.Y.Hessler, and K.R. Madden (1985).
- 25 *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 5, 3376-3385.

Example 1

Autonomous replication of plasmid p19R1 in *C. guilliermondii*

30

- [0021] The plasmid pFR1 carrying the *RIB1* gene of *C. guilliermondii* has been described (Zakalsky et al. Genetika 26, 614-620, 1990). In order to subclone the *RIB1* gene, plasmid pFR1 was digested with the restriction nuclease *Sall*. The resulting fragments were cloned into the *Sall* site of the pUC19 vector. The ligation mixture was transformed into the *E. coli* mutant strain BSV821 carrying a mutation of the *ribA* gene conducting to riboflavin deficiency. Colonies growing
- 35 in the absence of riboflavin were isolated and were shown to contain a plasmid p19R1.

- [0022] The plasmid p19R1 was sequenced and was shown to contain a 2.18 kb fragment of *C. guilliermondii* DNA in the *Sall* site of the pUC19 vector. The sequence of this insert is shown in Fig. 2.

- [0023] The DNA sequence shown in Fig. 2 carries the *RIB1* gene of *C. guilliermondii*. The plasmid transforms *C. guilliermondii* mutant defective in the *RIB1* gene to riboflavin prototrophy and can replicate autonomously in this yeast species. The replication was shown to be due to the presence of an autonomously replicating sequence (ARS) comprising approximately base pairs 1542 to 1755 in Fig. 2 and extending into the structural gene *RIB1*.
- 40

rib1 →
p19R1

Example 2

- 45 **Construction of a plasmid for hyperexpression of the *RIB1* gene of *C. guilliermondii***

- [0024] The *TEF* gene of the yeast, *S. cerevisiae*, specifies the translation elongation factor 1-alpha. This gene is known to be transcribed in *S. cerevisiae* with high efficiency.

- 50 [0025] A DNA fragment carrying the *S. cerevisiae* *TEF* promoter and the 5' part of the the *RIB1* gene of *C. guilliermondii* was obtained by PCR amplification. Initially, a DNA sequence located upstream from the 5' end of the *S. cerevisiae* *TEF* gene was amplified by PCR with primers ShBle_V and TEF1_H using chromosomal DNA of *S. cerevisiae* as a template. The amplified DNA fragment (subsequently designated *TEF* promoter) comprises bp 15 984 to 16 344 of the sequence listed under EMBL accession number gb/U51033/YSCP9513.

- [0026] Independently, a DNA fragment comprising the 5'-terminal part of *C. guilliermondii* GTP cyclohydrolase II structural gene was obtained by PCR with primers PGgtpCY_V and PGgtpCY_nco using p19R1 plasmid as a template. The amplified DNA fragment (subsequently designated 5'GTPcII) comprises bp 460 to 1145 of the sequence shown in Fig. 2.
- 55

- [0027] The DNA amplificates obtained in the two PCR reactions described above comprising parts of the *TEF* gene

of *S. cerevisiae* and of the 5'GTPcII were mixed, and a third PCR amplification was performed using the primers ShBle_V and PGgtpCY_nco. This reaction yielded a DNA fragment which contains the complete *TEF* promoter and the 5' part of the *C. guilliermondii* *RIB1* gene.

[0028] All primers used in these PCRs are shown in Table 2. The sequence of the final amplificate is shown in Fig. 3.

[0029] The amplificate contains a cutting site for the restriction nucleases *SphI* which had been introduced via the primer ShBle_V. The amplificate also contains an *MscI* site which is a feature of the *RIB1* gene. The amplificate was digested with *SphI* and *MscI*.

[0030] The plasmid p19R1 (whose construction is described above) was also digested with the same enzymes, and the PCR amplificate was ligated into the digested plasmid.

[0031] The ligation mixture was transformed into a mutant designated Rib7 of *Escherichia coli* carrying a mutation of the *ribA* gene which specifies GTP cyclohydrolase II. Transformation was performed by electroporation according to the protocol of Invitrogen (5).

[0032] The *E. coli* cells were plated on Luria-Broth plates supplemented with ampicillin (100 mg/ml) which did not contain riboflavin. Colonies growing on this medium were isolated and were shown to contain a plasmid designated pTC2.

[0033] The sequence of the insert of the plasmid pTC2 is shown in Fig. 3.

[0034] Plasmid pTC2 was digested with *XhoI* and *Sall* restriction endonucleases yielding 4.4 kb and 0.5 kb fragments. The 4.4 kb fragment was circularized with T4 DNA ligase. The ligation mixture was transformed into the Rib7 mutant of *E. coli* carrying the *RibA* mutation. Transformation was achieved by electroporation. The cells were plated on Luria-Broth plates containing ampicillin and no riboflavin (see above). Colonies growing on these plates were isolated and were shown to contain a plasmid pTCdXS2. The procedure had resulted in the removal of 0.5 kb base pairs from the plasmid pTC2.

[0035] The sequence of the insert of the plasmid pTCdXS2 is shown in Fig. 4. The open reading frame of the *RIB1* gene of *C. guilliermondii* is indicated. The DNA segment representing the promoter of the *TEF* gene of *S. cerevisiae* is also shown.

Example 3

Construction of a recombinant *C. guilliermondii* strains

[0036] The riboflavin deficient mutant rh-21 with an apparent defect of the *RIB1* gene specifying GTP cyclohydrolase II (2) has been obtained after chemical mutagenesis of the L2 strain (2) which was previously obtained from the ATCC 9058 *C. guilliermondii* strain.

[0037] The plasmid pTCdXS2 was transformed into the *RIB1* mutant strain rh-21 of *C. guilliermondii* by the LiCl procedure, respectively (6). The cells were plated on YPD medium without added riboflavin. Colonies growing without riboflavin were isolated. They were monitored for GTP cyclohydrolase II activity and for riboflavin production as described below.

[0038] The prototrophic strains were monitored for the presence of DNA segments introduced with the plasmid by PCR analysis. PCR was performed using the primers ShBle_V and PGgtpCY_nco and boiled *C. guilliermondii* recombinant strains cells as template. Primer ShBle_V is complementary to the *TEF* promoter and primer PGgtpCY_nco is complementary to the *RIB1* structural gene. Amplificates of the expected length (1175 base pairs) were obtained from all transformants isolated. The amplificate obtained from strain XS-3 was isolated and was sequenced by the fluorescent dideoxy terminator method. The sequence is shown in Fig. 5. This sequence is identical with base pairs 1 to 1168 of the insert of plasmid pTCdXS2.

[0039] The recombinant transformants were genetically stable. Specifically, they did not segregate riboflavin deficient subclones.

Example 4

GTP cyclohydrolase activity in recombinant *C. guilliermondii* strains

[0040] The level of GTP cyclohydrolase II activity in the recombinant strains described above was determined as follows.

[0041] The recombinant *C. guilliermondii* cells were grown aerobically in synthetic Burkholder medium supplemented with trace elements (Science 101, 180, 1945) but without asparagine, during 2-3 days at 30°C. *C. guilliermondii* L2 strain (wild type) served as a control in these experiments.

[0042] Cells from exponential growth phase were harvested by centrifugation (5000 g, 15 min), washed twice with 20 mM Tris HCl, pH 8.2, containing 1 mM DTT and 1 mM MgCl₂. Cells were stored at -20°C. Frozen cells mass (1-3 g) was thawed in 3-9 ml of washing buffer. Cells were disrupted by agitation with glass beads (d = 0.8 mm). After centrifugation,

cell extract was dialyzed overnight against 100 volumes of washing buffer. Protein concentration was measured by the Lowry method.

[0043] Reaction mixtures for GTP cyclohydrolase assays contained 20 mM Tris HCl, pH 8.2, 3 mM DTT, 2 mM MgCl₂, 1 mM GTP, and protein (protein concentration, 1-3 mg/ml, total volume, 4 ml). They were incubated at 37°C for 20 min in the dark.

[0044] After incubation, 2 ml aliquots were removed, and 2,3-butanedione was added to a final concentration of 0.5 mg/ml. The mixtures were incubated at 95°C for 30 min. Blank values were processed in the same way but without added diacetyl.

[0045] Differences in specific fluorescence of both types of aliquots were determined and were used to calculate the concentrations of 6,7-dimethylpteridin and the activity of GTP cyclohydrolase II. Results are shown in Table 3.

[0046] Strain L2 from which the mutant rh-21 had been derived was used as a control. The enzyme activity in strain L2 was 2,9 nmol mg⁻¹ h⁻¹. No enzyme activity was found in the riboflavin deficient recipient strain rh-21 carrying a mutation of the *RIB1* gene. The recombinant strains obtained by transformation with plasmid pTCdXS2 showed enzyme levels between 6,5 and 13,4 nmol mg⁻¹ h⁻¹. Thus, the enzyme level in recombinant strains was 2.3 - 4.6-fold higher as compared with the *C. guilliermondii* strain L2.

[0047] The activity of riboflavin synthase was also measured in the recombinant strains. The activity of riboflavin synthase was not affected by the transformation with the plasmids p19RI, pTC2, pTCdXS2. All strains analyzed had riboflavin synthase activities in the range of 20 nmol mg⁻¹ h⁻¹ (Table 3).

rib1 → pg

rib 7
pg +

Example 5

Production of riboflavin by recombinant *C. guilliermondii* strains

[0048] *C. guilliermondii* strains (wild type and recombinant strains) were grown aerobically in synthetic Burkholder medium supplemented with trace elements [Science 101, p. 180, (1945)] but without asparagine during 4 days at 30 °C [F.W. Tanner, Jr.C. Vojnovich, J.M. Van Lanen, Riboflavin production by Candida species. Nature, 1945, 101 (2616): 180-181]. The suspension was centrifuged. Riboflavin concentration was determined fluorometrically. Results are shown in Table 4.

[0049] The wild-strain L2 produced 1.2 mg riboflavin per liter under the conditions described. The recombinant strain XS3 produced a 3-fold increased level of riboflavin (3.6 mg/l).

pichia

Example 6

Isolation of riboflavin

[0050] Two Erlenmeyer flasks (2.5 l) each containing 0.5 l of synthetic Burkholder medium containing trace elements but no asparagine were inoculated with the recombinant *C. guilliermondii* strain XS-3. The cultures were incubated with shaking at 30°C for 50 h. The solution was centrifuged. The supernatant was passed through a column of Florisil (4 ml bed volume) at a velocity of 500 ml/h. The column was washed with distilled water (7 ml). Riboflavin was eluted by a mixture of acetone/1M aqueous NH₄OH. The effluent was evaporated to dryness. The yield of riboflavin was determined photometrically.

pichia

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[0051]

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Table 1

Enzymes and genes of the riboflavin pathway				
	Enzyme	Gene		
		<i>S. cerevisiae</i>	<i>C. guilliermondii</i>	<i>E. coli</i>
A	GTP cyclohydrolase	<i>RIB1</i>	<i>RIB1</i>	<i>ribA</i>
B	bacterial deaminase			<i>ribD</i>
C	yeast reductase	<i>RIB7</i>	<i>RIB2</i>	
D	yeast deaminase	<i>RIB2</i>	<i>RIB3</i>	
E	bacterial reductase			<i>ribD</i>
F	unknown phosphatase			
G	lumazine synthase	<i>RIB4</i>	<i>RIB5</i>	<i>ribE</i>
H	riboflavin synthase	<i>RIB5</i>	<i>RIB7</i>	<i>ribC</i>
I	3,4-dihydroxy-2-butanon 4-phosphate synthase	<i>RIB3</i>	<i>RIB6</i>	<i>ribB</i>

Table 2

Nucleotide sequences of the primers used.		
N	Primer	Sequence (5' - 3')
1	ShBle_V	GGGCATGCAATTCGAGCTCGGTACCCG
2	TEB1_H	CGACTCACTATAGGAGGAAGCTTGGCGC
3	PGgtpCY_V	AGGAGGAAGCTTGGCGCTATGGCATCGAAGG
4	PGgtpCY_nco	GCTGGTCGGTTAATGGGTGAAGCTGGG

Table 3

Activity of GTP cyclohydrolase II and riboflavin synthase in <i>C. guilliermondii</i> recombinant strains (time of growth: 40-48 h).				
N	Strain	Riboflavin synthase activity nmol mg ⁻¹ h ⁻¹	GTP cyclohydrolase II activity nmol mg ⁻¹ h ⁻¹	Ratio*
1	L2 (wild type)	21.6	2.88	1.00
2	R1-1	n.d.	10.08	3.50
3	R1-2	n.d.	4.20	1.46
4	R1-3	20.4	8.76	3.04
5	R1-4	19.8	7.80	2.70
6	R1-5	21.6	7.80	2.70
7	TC-1	20.4	9.60	3.33
8	TC-2	n.d.	8.40	2.92
9	TC-3	n.d.	7.56	2.63
10	XS-1	22.8	13.38	4.60
11	XS-2	n.d.	12.60	4.37
12	XS-3	n.d.	6.60	2.29

n.d. not determined.

* GTP cyclohydrolase activity of recombinant strain divided by GTP cyclohydrolase activity of strain L2

Table 4

Riboflavin production by recombinant <i>C. guilliermondii</i> strains (time of growth: 110 h. incubation temperature: 30 °C).			
N	Strain	Riboflavin production [mg/l]	Relative riboflavin production
1	L2 (wild type)	1.2	1.0
4	R1-3	1.4	1.2
5	R1-4	3.6	3.0
6	R1-5	3.0	2.5
7	TC-1	1.4	1.2
9	TC-3	1.3	1.0
12	XS-3	3.6	3.0
13	XS-4	2.0	1.7
14	XS-5	2.3	1.9

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<120> Overproduction of riboflavin in yeast

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Claims

1. A yeast strain which has been transformed by a recombinant DNA sequence comprising a DNA sequence which upon expression in a suitable host cell encodes at least one polypeptide with riboflavin biosynthetic activity and which DNA sequence is transcriptionally linked to a promotor functional in such yeast strain.
2. A yeast strain of claim 1 which belongs to the group of flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation.
3. A yeast strain of claim 2 which is selected from the following group: Schwanniomyces, preferably Schwanniomyces occidentalis, Debaryomyces, preferably Debaryomyces hansenii, Torulopsis, preferably Torulopsis candida, or Candida, preferably Candida guilliermondii or Candida famata.
4. The yeast strain of claim 3 which is Candida guilliermondii or Candida famata.
5. A yeast strain as claimed in any one of claims 1 to 4 wherein the polypeptide encoding DNA sequence is from yeast, preferably flavinogenic yeasts which overproduce riboflavin under conditions of iron starvation more preferably Candida, e.g. Candida guilliermondii or Candida famata.
6. A yeast strain as claimed in any one of claims 1 to 4, wherein the polypeptide encoding DNA sequence encodes a

protein with GTP cyclohydrolase II activity and is selected from the following DNA sequences:

a) the DNA sequence as shown in Fig. 5 or its complementary strand;

5 b) DNA sequences which hybridize under standard conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and

c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

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7. A yeast strain as claimed in any one of claims 1 to 6 wherein the promotor is the TEF *S. cerevisiae* promotor.

8. A process for the production of riboflavin characterized therein that a yeast strain as claimed in any one of claims 1 to 7 is cultured under suitable culture conditions and the riboflavin produced is isolated from the medium or the yeast strain by methods known to the man skilled in the art.

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9. A process for the production of a food or feed composition characterized therein that riboflavin as obtained by the process of claim 8 is mixed with one or more suitable food or feed ingredients.

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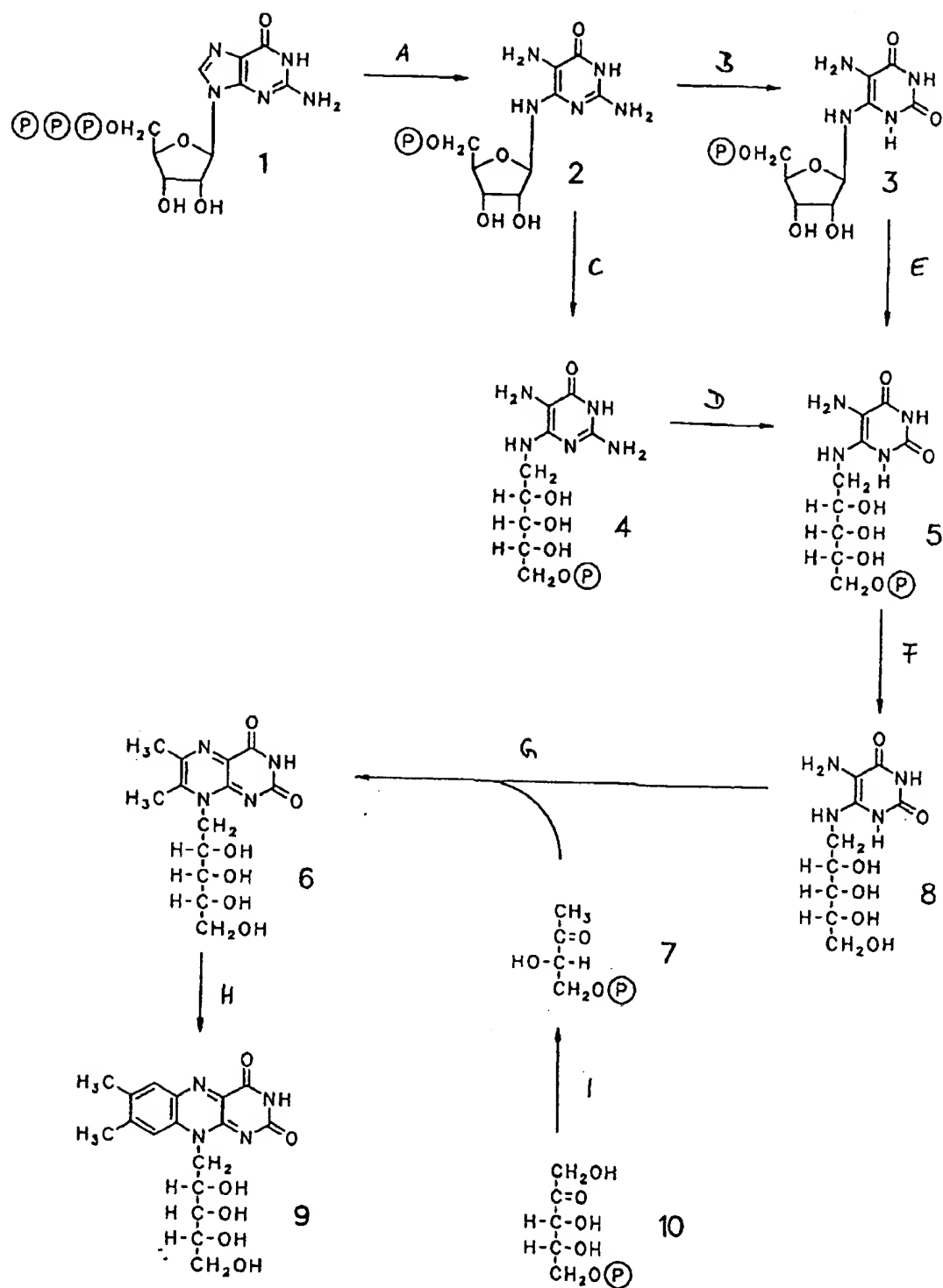


Fig. 1

Fig.2.
Nucleotide sequence of the p19R1 insert.

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```

      490     500     510     520     530     540
      |      |      |      |      |      |
CGAAGGACATAGTACATCCGCAACCAGAGCGCCGGCACGGGTCGGAACTCACGAATTTA

```

LysAspIleValHisProGlnProGluArgArgHisGlySerGluThrHisGluPheThr

```

      550     560     570     580     590     600
      |      |      |      |      |      |
CCATGCCTCTCTTATCTCCTACATTGACACCATCCCATATTCCATCGCAAACGCCTCAAA

```

METProLeuLeuSerProThrLeuThrProSerHisIleProSerGlnThrProGlnIle

610 620 630 640 650 660
 TTCCTCCGGAAGTCCAGCAGAAGTCAGGGATCGCTTGCCCTTCCTGAAACGTTGCCTG
 ProProGluValProAlaGluValArgAspArgLeuProLeuProGluThrLeuProVal
 670 680 690 700 710 720
 TGGTGAAATGCATGGCGAGAGCTCGTATACCGACCACTCAGGGGCCGAGATATTCTCC
 ValLysCysMETAlaArgAlaArgIleProThrThrGlnGlyProGluIlePheLeuHis
 730 740 750 760 770 780
 ATTTGTACGAGAATAACGTTGACAATAAAGAGCATTGGCTATTGTTTTGGGGAAGATG
 LeuTyrGluAsnAsnValAspAsnLysGluHisLeuAlaIleValPheGlyGluAspVal
 790 800 810 820 830 840
 TGCGGTGCAAAACGCTCTATCAGAAACGTCCCAATGAGACCCAGCAAGATAGAATGACTC
 ArgSerLysThrLeuTyrGlnLysArgProAsnGluThrGlnGlnAspArgMETThrArg
 850 860 870 880 890 900
 GTGGTGCTTATGTGGGCAGATTGTTTCCTGGAAGAACCGAGGCAGACTATGACAGTGAGT
 GlyAlaTyrValGlyArgLeuPheProGlyArgThrGluAlaAspTyrAspSerGluSer
 910 920 930 940 950 960
 CTAATTTGAGATTGAATTTTCGATGAAAATGGCCAATTATCAGAGATCCGAGTACCACCT
 AsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeuIleArgAspProSerThrThrCys
 970 980 990 1000 1010 1020
 GTAGTGGTGAGCCCATTTTGCCCGTATTCATTGGAATGTTATACGGGGGAAACCGCAT
 SerGlyGluProIleLeuAlaArgIleHisSerGluCysTyrThrGlyGluThrAlaTrp
 1030 1040 1050 1060 1070 1080
 GGAGTGCTCGTTGCGATTGTGGAGAACAATTCGATGAAGCTGGTCGGTTAATGGGTGAAG
 SerAlaArgCysAspCysGlyGluGlnPheAspGluAlaGlyArgLeuMETGlyGluAla

1090 1100 1110 1120 1130 1140
 | | | | | |
 CTGGGCACGGGTGTATCGTGTACCTTCGTCAGGAAGGTCGTGGAATTGGACTTGGGGAAA
 GlyHisGlyCysIleValTyrLeuArgGlnGluGlyArgGlyIleGlyLeuGlyGluLys

1150 1160 1170 1180 1190 1200
 | | | | | |
 AGTTGAAGGCTTATAATTGCAAGACTTGGGAGCGGATACCGTCCAGGCCAATTGATGT
 LeuLysAlaTyrAsnLeuGlnAspLeuGlyAlaAspThrValGlnAlaAsnLeuMETLeu

1210 1220 1230 1240 1250 1260
 | | | | | |
 TACGACATCCTGCTGATGCGAGATCTTTTCGCTCGCTACAGCCATACTCTTGGACTTGG
 ArgHisProAlaAspAlaArgSerPheSerLeuAlaThrAlaIleLeuLeuAspLeuGly

1270 1280 1290 1300 1310 1320
 | | | | | |
 GGCTCAACGAGATCAAGTTGTTGACCAACAATCCCGATAAAATTGCTGCAGTTGAGGGAA
 LeuAsnGluIleLysLeuLeuThrAsnAsnProAspLysIleAlaAlaValGluGlyArg

1330 1340 1350 1360 1370 1380
 | | | | | |
 GAAACAGAGAGGTCAAGGTAGTGGAACGGGTGCCTATGGTGCCGTGGCATGGAGAACTG
 AsnArgGluValLysValValGluArgValProMETValProLeuAlaTrpArgSerGlu

1390 1400 1410 1420 1430 1440
 | | | | | |
 AGAATGGAATCAAGTCAAAGAGATAGAGGGCTACTTGAGTGCTAAGATTGAAAGGATGG
 AsnGlyIleLysSerLysGluIleGluGlyTyrLeuSerAlaLysIleGluArgMETGly

1450 1460 1470 1480 1490 1500
 | | | | | |
 GGCACCTTGCTTGAAGCCACTCAAGATATGATAGAAGAGATGAAGTTAAGGACTTAAGA
 HisLeuLeuGluLysProLeuLysIle-----

1510 1520 1530 1540 1550 1560
 | | | | | |
 AATAAATGATGAATTAAATGACGCAAATGTCACCTACTCGATTAGAGAAATAGCTATAATG

1570 1580 1590 1600 1610 1620
 | | | | | |
 AAGAATTTTGCATTTTCGCAAAATTTAAGATAAATGCAAAAATTGCAAATTACGAAATATG

butsky
butsky top : ARS number

1630 1640 1650 1660 1670 1680
 CATATGATACAAGACAAGAAAAGACTACTAAAA**GTCTCTCGAGAAGAATACTGGGTAACC**

1690 1700 1710 1720 1730 1740
 TTCATCTCTTGATTATGCACTGGGGCTATT**CATATGCAGATTTCGCACGCCGAGGTGCAGC**

1750 1760 1770 1780 1790 1800
 GTTTAGGCGCGGCTCAACGGAAGCCAACGGCCGCCACAAATTGTCCGGAAAGTCGCCGAA

1810 1820 1830 1840 1850 1860
 ACTGATCCACTGGTAC**CACAGCCCCATAAGAACCCCTTTAATATTAAACCGTTCTTC**

1870 1880 1890 1900 1910 1920
 AGCCACTTTTGATCACATTGTTTGCAGCCGCCCGTTGCTGCCATCCAAACACCACGCGTC

1930 1940 1950 1960 1970 1980
 CCCCCGACCTTTTACGGTGCCCACTGCATTGGAATTTGCATAAAACAGCCTCACGAAGTG

1990 2000 2010 2020 2030 2040
 GATTAATTTTATAGCACTCAAGTCATCATCTGCAATCTCTGCATCATGAAATGACTCC

2050 2060 2070 2080 2090 2100
 CGTTGATACAGGGAAC**TCAGACCGCAAGCGCGAAGAGTCACAAGAGCGTGTGATGTGTG**

2110 2120 2130 2140 2150 2160
 TCGACTCTAGAGATCCCCGGTAC**GAGCTCGAATTC**ACTGGCCGTCGTTTACACGTC

2170 2180
 GTGACTGGGAAAACCCCTGGCG

Sequence of the *RIB1* gene is translated.

Sequence of ARS element is typed in bold letters.

Fig.3
Nucl otide sequence of the pTC2 insert.

```

      10      20      30      40      50      60
      |      |      |      |      |      |
CAATTCGAGCTCGGTACCCGGGATCCCCACACACCATAGCTTCAAAATGTTTCTACTC

      70      80      90     100     110     120
      |      |      |      |      |      |
CTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC

     130     140     150     160     170     180
     |      |      |      |      |      |
CAAGCACAGCATACTAAATTTCCCTCTTTCTTCTCTAGGGTGTCGTTAATTACCCGTAC

     190     200     210     220     230     240
     |      |      |      |      |      |
TAAAGGTTTGGAAAAAGAAAAAGAGACCGCCTCGTTTCTTTTCTTCGTCGAAAAAGGCA

     250     260     270     280     290     300
     |      |      |      |      |      |
ATAAAAATTTTATCACGTTTCTTTTTCTTGAAATTTTITTTTGGATTTTTTCTCTTI

     310     320     330     340     350     360
     |      |      |      |      |      |
CGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTTCTCAAGTTTCAGTT

     370     380     390     400     410     420
     |      |      |      |      |      |
TCATTTTTCTTGTTCTATTACAACTTTTTTACTTCTTGCTCATTACAAAGAAACATAG

     430     440     450     460     470     480
     |      |      |      |      |      |
CAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACCGGGCGCCT

     490     500     510     520     530     540
     |      |      |      |      |      |
ACTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAG

     550     560     570     580     590     600
     |      |      |      |      |      |
- GAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGCGCCGCA

```

METAlaSerLy^sTyrIleValHisProGlnProGluArgArgHis

610 620 630 640 650 660
 CGGGTCGGAACTCAGCAATTTACCATGCCTCTCTTATCTCCTACATTGACACCATCCCA
 GlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrProSerHis
 670 680 690 700 710 720
 TATTCCATCGCAAACGCCCTCAAATTCCTCCCGAAGTCCCAGCAGAAGTCAGGGATCGCTT
 IleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAspArgLeu
 730 740 750 760 770 780
 GCCCCCTTCCTGAAACGTTGCCCTGTGGTGAAATGCATGCCGACAGCTCCTATACCGACCAC
 ProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIleProThrThr
 790 800 810 820 830 840
 TCAGGGGCCGGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAGAGCATT
 GlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGluHisLeu
 850 860 870 880 890 900
 CGCTATTGTTTTTGGGAAGATGTGCGGTGCGAAAACGCTCTATCAGAAACGTCCCAATGA
 AlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgProAsnGlu
 910 920 930 940 950 960
 GACCCAGCAACATAGAATCACTCGTGGTGCTTATCTGGGCAGATTGTTTCCTGGAAGAAC
 ThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGlyArgThr
 970 980 990 1000 1010 1020
 CGAGGCAGACTATGACAGTGAGTCTAATTTGACATTGAATTTGATGAAAATGGCCAACT
 GluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeu
 1030 1040 1050 1060 1070 1080
 TATCAGAGATCCGAGTACCACCTGTAOTGGTGAGCCCATTTTGGCCCGTATTCATTCCGA
 IleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHisSerGlu

Fig.4
Nucleotide sequence of the pTCdXS-2 insert.

```

      10      20      30      40      50      60
      |      |      |      |      |      |
CAATTCGAGCTCGGTACCCGGGGATCCCCACACACCATAGCTTCAAAATGTTTCTACTC

      70      80      90     100     110     120
      |      |      |      |      |      |
CTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC

     130     140     150     160     170     180
     |      |      |      |      |      |
CAAGCACAGCATACTAAATTTCCCTCTTCTTCCTCTAGGGTGTCGTTAATTACCCGTAC

     190     200     210     220     230     240
     |      |      |      |      |      |
TAAAGGTTTGAAAAAGAAAAAGAGACCGCCTCGTTTCTTTTCTTCGTCGAAAAAGGCA

     250     260     270     280     290     300
     |      |      |      |      |      |
ATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTGATTTTTTCTCTTT

     310     320     330     340     350     360
     |      |      |      |      |      |
CGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTCTCAAGTTTCAGTT

     370     380     390     400     410     420
     |      |      |      |      |      |
TCATTTTCTTGTTCATTACAACCTTTTTTACTTCTTGCTCATTAGAAAAGAAAGCATAG

     430     440     450     460     470     480
     |      |      |      |      |      |
CAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACCGGCGCCT

     490     500     510     520     530     540
     |      |      |      |      |      |
AGTGTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAG

     550     560     570     580     590     600
     |      |      |      |      |      |
GAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGCGCCGGCA

```

METAlaSerLysTyrIleValHisProGlnProGluArgArgHis

610 620 630 640 650 660
 CGGGTCGGAACTCACGAATTACCATGCCTCTCTTATCTCCTACATTGACACCATCCCA
 GlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrProSerHis

670 680 690 700 710 720
 TATTCCATCGCAAACGCCTCAAATTCCTCCGGAAGTGCCAGCAGAAGTCAGGGATCGCTT
 IleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAspArgLeu

730 740 750 760 770 780
 GCCCCTTCCTGAAACGTTGCCTGTGGTGAAATGCATGGCGAGAGCTCGTATACCGACCAC
 ProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIleProThrThr

790 800 810 820 830 840
 TCAGGGGCCGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAGAGCATT
 GlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGluHisLeu

850 860 870 880 890 900
 GGCTATTGTTTTGGGGAAGATGTGCGGTCGAAAACGCTCTATCAGAAACGTCCCAATGA
 AlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgProAsnGlu

910 920 930 940 950 960
 GACCCAGCAAGATAGAATGACTCGTGGTGCTTATGTGGGCAGATTGTTTCCTGGAAGAAC
 ThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGlyArgThr

970 980 990 1000 1010 1020
 CGAGGCAGACTATGACAGTGAGTCTAATTTGAGATTGAATTCGATGAAAATGGCCAACT
 GluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGlyGlnLeu

1030 1040 1050 1060 1070 1080
 TATCAGAGATCCGAGTACCACCTGTAGTGGTGAGCCCATTTGGCCCGTATTTCATTCGGA
 IleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHisSerGlu

1090 1100 1110 1120 1130 1140
 ATGTTATACGGGGAAACCGCATGGAGTGCTCGTTGCGATTGTGGAGAACAAATTCGATGA
 CysTyrThrGlyGluThrAlaTrpSerAlaArgCysAspCysGlyGluGlnPheAspGlu

1150 1160 1170 1180 1190 1200
 AGCTGGTCGGTTAATGGGTGAAGCTGGGCACGGGTGTATCGTGTACCTTCGTCAGGAAGG
 AlaGlyArgLeuMETGlyGluAlaGlyHisGlyCysIleValTyrLeuArgGlnGluGly

1210 1220 1230 1240 1250 1260
 TCGTGGAAATTGGACTTGGGGAAAAGTTGAAGGCTTATAATTTGCAAGACTTGGGAGCGGA
 ArgGlyIleGlyLeuGlyGluLysLeuLysAlaTyrAsnLeuGlnAspLeuGlyAlaAsp

1270 1280 1290 1300 1310 1320
 TACCGTCCAGGCCAATTTGATGTTACGACATCCTGCTGATGCGAGATCTTTTTCGCTCGC
 ThrValGlnAlaAsnLeuMETLeuArgHisProAlaAspAlaArgSerPheSerLeuAla

1330 1340 1350 1360 1370 1380
 TACAGCCATACTCTTGGACTTGGGGCTCAACGAGATCAAGTTGTTGACCAACAATCCCGA
 ThrAlaIleLeuLeuAspLeuGlyLeuAsnGluIleLysLeuLeuThrAsnAsnProAsp

1390 1400 1410 1420 1430 1440
 TAAAATTGCTGCAGTTGAGGGAAGAAACAGAGAGGTCAAGGTAGTGGAAACGGGTGCCTAT
 LysIleAlaAlaValGluGlyArgAsnArgGluValLysValValGluArgValProMET

1450 1460 1470 1480 1490 1500
 GGTGCCGTTGGCATGGAGAAGTGAGAATGGAATCAAGTCAAAAGAGATAGAGGGCTACTT
 ValProLeuAlaTrpArgSerGluAsnGlyIleLysSerLysGluIleGluGlyTyrLeu

1510 1520 1530 1540 1550 1560
 GAGTGCTAAGATTGAAAGGATGGGGCACTTGCTTGAAAAGCCACTCAAGATATGATAGAA
 SerAlaLysIleGluArgMETGlyHisLeuLeuGluLysProLeuLysIle-----

1570	1580	1590	1600	1610	1620
GAGATGAAGTTAAGGACTTAAGAAATAAATGATGAATTAAATGACGCAAATGTC ACTACT					
1630	1640	1650	1660	1670	1680
CGATTAGAGAAATAGCTATAATGAAGAATTTTGCATTTTCGCAAAATTTAAGATAAATGCA					
1690	1700	1710	1720	1730	1740
AAAATTGCAAATTACGAAATATGCATATGATACAAGACAAGAAAAGACTACTAAAAGTCTCT					

Sequence of the *TEF* promoter is underlined.
 Sequence of the *RIB1* gene is translated.
 Sequence of ARS element is typed in bold letters.

Fig.5

Nucleotide sequence of PCR product obtained with primers ShBle_V and PGgtpCY_nco using boiled cells of *P.guilliermondii* XS-3 recombinant strain.

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      10      20      30      40      50      60
      |      |      |      |      |      |
GGGCATGCAATTCGAGCTCGGTACCCGGGGATCCCCACACACCATAGCTTCAAAATGTT

      70      80      90     100     110     120
      |      |      |      |      |      |
TCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCA

      130     140     150     160     170     180
      |      |      |      |      |      |
AAACACCCAAGCACAGCATACTAAATTTCCCTCTTCTTCTCTAGGGTGTCTTAATTA

      190     200     210     220     230     240
      |      |      |      |      |      |
CCCGTACTAAAGGTTTGGAAAAGAAAAAGAGACCGCCTCGTTTCTTTTCTTCGTCGAA

      250     260     270     280     290     300
      |      |      |      |      |      |
AAAGGCAATAAAAAATTTTATCACGTTTCTTTTCTTGAAATTTTTTTTTTGATTTTTT

      310     320     330     340     350     360
      |      |      |      |      |      |
TCTCTTTCGATGACCTCCCATTGATATTTAAGTCAATAAACGGTCTTCAATTTCTCAAGT

      370     380     390     400     410     420
      |      |      |      |      |      |
TTCAGTTTCATTTTTTCTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAA

      430     440     450     460     470     480
      |      |      |      |      |      |
AGCATEGCAATCTAATCTAAGGGCGAGCTCGAATTCGAACTAGTACTGCAGCACGTGACC

      490     500     510     520     530     540
      |      |      |      |      |      |
GGCGCCTAGTGTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTC

      550     560     570     580     590     600
      |      |      |      |      |      |
ACTATAGGAGGAAGCTTGGCGCTATGGCATCGAAGTACATAGTACATCCGCAACCAGAGC

```

METAlaSerLysTyrIleValHisProGlnProGluArg

610 620 630 640 650 660
 | | | | | |
 GCCGGCACGGGTCGGAACTCACGAATTTACCATGCCTCTCTTATCTCCTACATTGACAC
 ArgHisGlySerGluThrHisGluPheThrMETProLeuLeuSerProThrLeuThrPro

670 680 690 700 710 720
 | | | | | |
 CATCCCATATTCCATCGCAAACGCCTCAAATTCCTCCGGAAGTGCCAGCAGAAGTCAGGG
 SerHisIleProSerGlnThrProGlnIleProProGluValProAlaGluValArgAsp

730 740 750 760 770 780
 | | | | | |
 ATCGCTTGCCCTTCCTGAAACGTTGCCGTGGTGAAATGCATGGCGAGAGCTCGTATAC
 ArgLeuProLeuProGluThrLeuProValValLysCysMETAlaArgAlaArgIlePro

790 800 810 820 830 840
 | | | | | |
 CGACCACTCAGGGCCGGAGATATTTCTCCATTTGTACGAGAATAACGTTGACAATAAAG
 ThrThrGlnGlyProGluIlePheLeuHisLeuTyrGluAsnAsnValAspAsnLysGlu

850 860 870 880 890 900
 | | | | | |
 AGCATTGCGCTATTGTTTTGGGAAGATGTGCGGTCGAAAACGCTCTATCAGAAACGTC
 HisLeuAlaIleValPheGlyGluAspValArgSerLysThrLeuTyrGlnLysArgPro

910 920 930 940 950 960
 | | | | | |
 CCAATGAGACCCAGCAAGATAGAATGACTCGTGGTGCTTATGTGGGCAGATTGTTTCCTG
 AsnGluThrGlnGlnAspArgMETThrArgGlyAlaTyrValGlyArgLeuPheProGly

970 980 990 1000 1010 1020
 | | | | | |
 GAAGAACCGAGGCAGACTATGACAGTGAGTCTAATTGAGATTGAATTTGATGAAAATG
 ArgThrGluAlaAspTyrAspSerGluSerAsnLeuArgLeuAsnPheAspGluAsnGly

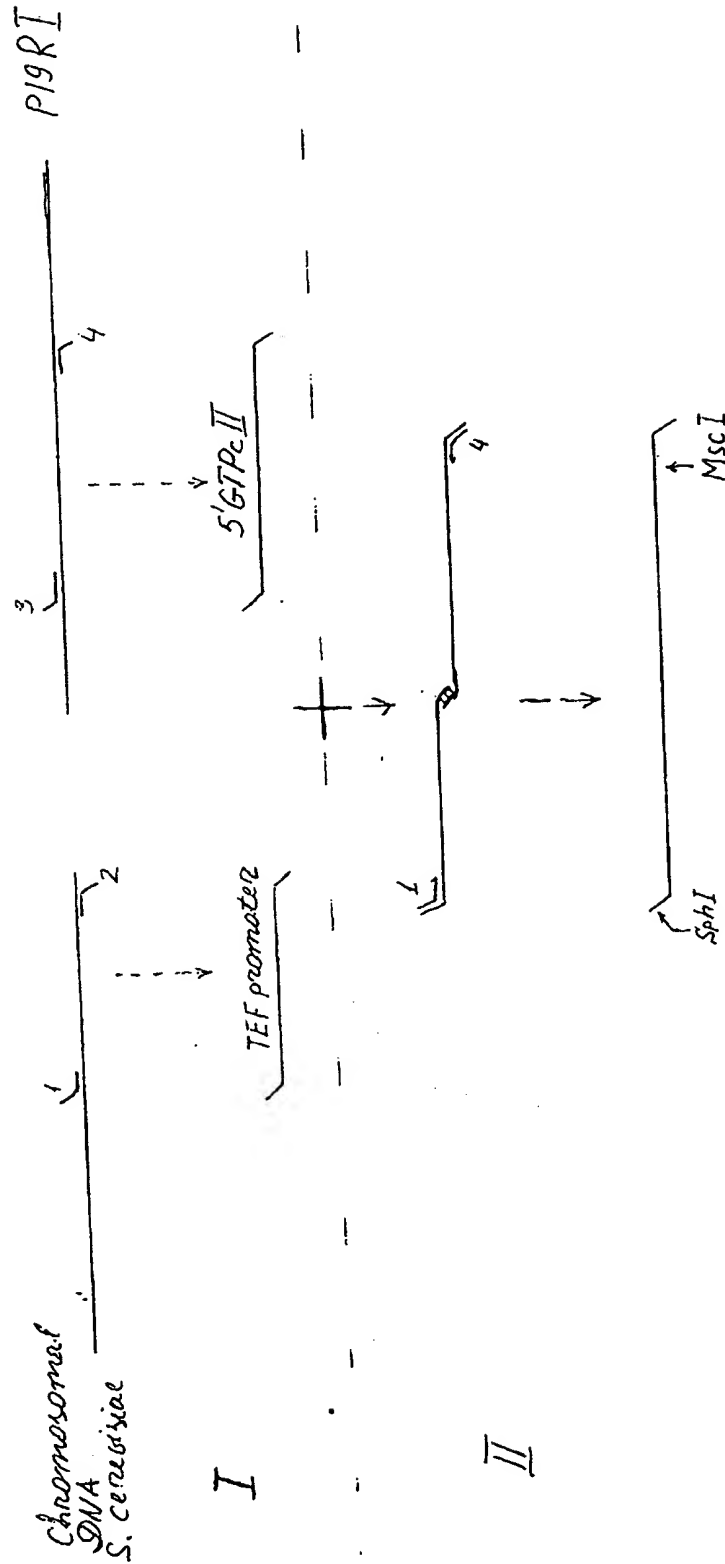
1030 1040 1050 1060 1070 1080
 | | | | | |
 GCCAACTTATCAGAGATCCGAGTACCACCTGTAGTGGTGAGCCCATTTGGCCCGTATTC
 GlnLeuIleArgAspProSerThrThrCysSerGlyGluProIleLeuAlaArgIleHis

1090 1100 1110 1120 1130 1140
 | | | | | |
 ATTCGGAATGTTATACGGGGGAAACCGCATGGAGTGCTCGTTGCGATTGTGGAGAACAAT
 SerGluCysTyrThrGlyGluThrAlaTrpSerAlaArgCysAspCysGlyGluGlnPhe

1150 1160 1170
 | | |
 TCGATGAAGCTGGTCGGTTAATGGGTGAAGCTGGG
 AspGluAlaGlyArgLeuMETGlyGluAlaGly

Sequence of the *TEF* promoter is underlined.
 Partial sequence of the *RIB1* gene is translated.

Scheme
of the 2-steps PCR amplification.



1. SkBle-V
2. TEF1-H
3. PGgtpcy-V
4. PGgtpcy-nc0